Gene Amplification in Esophageal Adenocarcinomas and Barrett’s with High-Grade Dysplasia

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ABSTRACT

Purpose: The purpose of this study was to determine the frequency and overall contribution of specific gene amplification events in the formation of Barrett’s adenocarcinomas. The relationship of gene amplification to clinical-pathological variables and its potential usefulness as a marker for early cancer detection were also examined.

Experimental Design: We used quantitative PCR and Southern blot analysis to screen 87 cases of Barrett’s adenocarcinoma for the presence or absence of 13 distinct gene amplification events. Gene amplification was then examined for correlation with other amplification events and clinical variables (survival, stage, nodal involvement, tumor invasion, smoking history, and gender). Additionally, 22 specimens of Barrett’s with high-grade dysplasia (HGD) were examined for the presence of gene amplification.

Results: One or more amplification events were present in 50 of 87 (57%) adenocarcinomas. The ERBB2 gene was amplified in 19 of 87 (21.8%), CCNE1 in 11 of 87 (12.6%), GATA4 in 9 of 87 (10.3%), KRAS in 9 of 87 (10.3%), EGFR in 7 of 87 (8.0%), CCND1 in 6 of 87 (6.8%), HNF3a in 5 of 87 (5.7%), PIK3CA in 5 of 87 (5.7%), C-MYC in 4 of 87 (4.6%), DYRK2 in 2 of 87 (2.3%), and AIB1, AKT1, and IGF1R were amplified in 0 of 87 (0%) of the tumors. CCND1 amplification was found to correlate negatively with survival (P < 0.05). In addition, the ERBB2 amplicon positively correlated (P < 0.05) with GATA4 amplification. Increased copy number of the ERBB2 (1 of 22), GATA4 (1 of 22), KRAS (2 of 22), C-MYC (1 of 22), CCNE1 (2 of 22), and CCND1 (2 of 22) genes was also observed in one or more Barrett’s adenocarcinomas with HGD.

Conclusions: The high frequency of gene amplification in esophageal adenocarcinomas and HGD indicates the important role of these events in esophageal adenocarcinoma development. Additionally, these results underscore the possible usefulness of early detection approaches and chemotherapeutic strategies (ErB2 and cyclin D1) targeted against amplified gene products.

INTRODUCTION

Barrett’s adenocarcinoma is associated with poor clinical outcome and 5-year survival rates of 5–15%, reflecting the high percentage of tumors with local invasion and distant metastases at diagnosis (1). Barrett’s metaplasia is an acquired condition caused by protracted gastroesophageal reflux and involves replacement of normal squamous epithelium with a columnar-lined mucosa (2). Although this condition is observed in only 10–12% of patients with gastroesophageal reflux disease, patients diagnosed with Barrett’s metaplasia have a 30- to 40-fold increased risk of developing adenocarcinoma compared with the general population (3, 4). Consequently, these patients undergo endoscopic surveillance to detect malignancies or Barrett’s mucosa with HGD, which portends coincident adenocarcinoma in 30–50% of patients (5–8).

Barrett’s adenocarcinoma develops from myriad genetic alterations. Mutations and allelic losses involving the p16INK4a and p53 tumor suppressor genes are frequently detected in Barrett’s adenocarcinomas and are also associated with dysplastic changes in premalignant Barrett’s epithelium (9, 10). Studies of other tumor suppressor genes have shown high-frequency allelic loss of the APC, Rb, MCC, and DCC genes in these tumors (11, 12). In addition to chromosomal deletions, amplification of cellular oncogenes plays a salient role in promoting neoplastic progression in many tumor types (13). Gene amplification is a poorly understood process that results in extra- and intrachromosomal gains of specific DNA sequences. Using restriction landmark genome scanning and Q-PCR, we successfully identified and characterized the 8p22 (CTSB/GATA4), 19q12 (CCNE1), 3q26 (PIK3CA), 12q14 (DYRK2), and 14q13 (HNF3a) amplification regions in Barrett’s adenocarcinomas (14–19). In addition, we and others have observed gene amplification to underlie a transcriptional up-regulation for the ERBB2, EGFR, CCND1, PIK3CA, and GATA4 genes (20, 21). The present study was aimed at determining the amplification status of many other known oncogenes in Barrett’s adenocarcinomas and the collective role of gene amplification in individual...
Barrett’s adenocarcinomas with regard to pathological and clinical variables. This will allow further understanding as to what extent gene amplification plays in promoting malignant behavior in this tumor type. We used Q-PCR and Southern blot analysis to examine the amplification frequency of commonly amplified genes in a series of 87 Barrett’s adenocarcinomas and 22 Barrett’s specimens with HGD. One or more amplification events were found in 57% of esophageal adenocarcinomas. Further analysis of genomic amplification with clinical variables identified CCND1 amplification as associated with unfavorable patient survival. Additionally, increased gene copies of ERBB2, CCND1, GATA4, C-MYC, and KRAS were observed in microdissected Barrett’s mucosa with HGD. These data support an important role for gene amplification in a plurality of tumors and suggest that gene amplification occurs before malignant transformation.

**MATERIALS AND METHODS**

**Tissue Processing and Procurement.** Patient consent was received, and the project was approved by the institutional review board of the University of Michigan Medical School. Tissues from 87 esophageal adenocarcinoma patients undergoing esophagectomy for malignancy and 22 patients undergoing esophagectomy for HGD at the University of Michigan Medical Center between 1991 and 2001 were collected and analyzed for this study. HGD specimens were initially diagnosed by two clinical pathologists, according to published criteria for HGD, from endoscopic biopsies and verified on resected tissues after surgery (22, 23). Patients receiving preoperative radiation or chemotherapy were excluded from this study. Tissues were transported to the laboratory on ice in DMEM. A representative portion of each specimen was embedded in OCT compound (Miles Scientific, Naperville, IL) for use in cryostat sectioning, and the remaining portion was frozen in liquid nitrogen for DNA isolation and stored at −80°C. Cryostat sections of all Barrett’s adenocarcinomas were analyzed, and only portions of tumors containing >70% tumor cellularity were used in this study. DNA was isolated from the esophageal adenocarcinoma and either the normal esophageal squamous mucosa or normal gastric mucosa from each patient. In addition, 10-μm frozen sections were used for microdissection and DNA extraction from HGD tissues removed from 22 patients. An esophageal cancer database, pathological records, and patient charts were reviewed for all clinical information.

**Cell Culture.** Esophageal squamous (Het-1A) and Barrett’s adenocarcinoma (Seg-1, Flo-1, and Bic-1) cell lines were used. The Seg-1, Flo-1, and Bic-1 cell lines were derived from human esophageal Barrett’s adenocarcinomas (24). Het-1A is a normal human esophageal mucosa cell line immortalized using SV40 and was described previously (25). Cells were grown in 10-cm tissue culture dishes with DMEM containing 10% fetal bovine serum and 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate antibiotics at 37°C in a 5% CO2 humidified atmosphere. Cells were collected by scraping with a rubber policeman and pelleted at 1200 rpm for 2 min. The medium was then removed, the cells were frozen immediately in liquid nitrogen, and pellets were stored at −70°C until processing for DNA and RNA isolation.

**DNA Isolation and Q-PCR.** DNA isolation and preparation were performed as described previously (15) Candidate genes and oncogenes mapping to known amplified regions were selected for Q-PCR, as described previously (15). Forward primers were end-labeled with [γ-32P]ATP (New England Nuclear Life Science Products, Boston, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). PCR was performed using Taq polymerase (Promega, Madison, WI), and the PCR products were resolved on 8% denaturing polyacrylamide gels. PCR product signal ratios (the intensity ratio of tumor (Ts/c) or normal (Ns/c) samples versus GAPDH control from Q-PCR (Ts/c:Ns/c)) for both the tumor (Ts/c, tumor tested fragment/tumor GAPDH fragment) and normal DNA samples (Ns/c, normal tested fragment/nomral GAPDH fragment) were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Amplification ratios of ≥2.0 were considered indicative of DNA amplification. All results were repeated and verified.

**Southern Blot Analysis.** For each sample, 10 μg of DNA were digested overnight at 37°C with 20 units of EcoRI (Promega), separated by electrophoresis in 0.8% agarose gels containing ethidium bromide, and then vacuum-blotted onto a GeneScreen Plus (New England Nuclear, Boston, MA) membrane with the LKB 2016 VacuGene blotting system. DNA probes included human ERBB2 (26), human EGFR (27), viral KRAS (28), human AIB1, (29), and mouse c-myc cDNA (30). Cloned cDNA inserts were excised from their plasmid vectors, purified, and labeled with [32P]dCTP using the random primer method (Life Technologies, Inc., Carlsbad, CA). The hybridized and washed membranes were exposed to Hyperfilm (Amersham, Piscataway, NJ) with intensifying screens from 4 to 19 days at −70°C. Band intensities from the autoradiographs were quantified by laser scanning densitometry using an ImageQuant Personal Densitometer (Molecular Dynamics, Piscataway, NJ). Blots were stripped of previously hybridized probes in 0.1× SSC, 1% SDS for 20 min at 95°C to allow rehybridization. Band intensity signals were corrected for differences in DNA loading and transfer by reprobing blots with CMKLRI (31). The degree of gene amplification was expressed as a ratio of the gene band density in the tumor divided by the gene band density in the patient’s normal tissue. A ratio of ≥2.0 was considered indicative of gene amplification.

**Real-Time PCR.** Taqman primers for GAPDH (Invitrogen, Carlsbad, CA) and GATA4 (Biosearch Technologies, Novato, CA) were optimized for Mg2+, primer concentrations, and reaction conditions. SYBR Green (Molecular Probes, Eugene, OR) was used as the reporter dye at a working concentration of 0.5×. All reactions were performed in a 25-μl volume using the Smart-Cycler Real-time PCR System (Cepheid, Sunnyvale, CA), and results were verified in triplicate. The second derivative of the standard growth curve was used to define its inflection point, allowing accurate detection of the transition from background to amplified product fluorescence.

**Statistical Analyses.** Cox proportional hazards regression methods were used to adjust for significant clinical variables before testing the significance of various amplicons. Fisher’s exact test was used to test for a relationship between the categorical clinical variables and both amplification as a whole and individual amplicons. Significant relationships between the
continuous clinical variables and the individual amplicons were tested using logistic regression, where the response was presence of amplification. To assess whether any of the individual amplicons were related to higher frequency of overall amplification, we used a Poisson model where the response is the number of positive amplicons excluding the current amplicon under consideration. Each amplicon was used individually as a predictor of this response.

RESULTS

Gene Amplification in Barrett’s Adenocarcinomas.
The amplification frequency of 13 candidate genes and oncogenes was examined in DNA from 87 Barrett’s adenocarcinoma patients with the use of Q-PCR. Representative amplification of ERBB2, EGFR, KRAS, C-MYC, CCND1, and DYRK2 is shown for a subset of the 87 adenocarcinomas examined (Fig. 1; Table 1). Amplification was observed in 50 of 87 (57%) tumors, which included the following: ERBB2, amplified in 19 of 87 (21.8%) tumors; CCNE1, amplified in 11 of 87 (12.6%) tumors; EGFR, amplified in 7 of 87 (8.0%) tumors; KRAS, amplified in 9 of 87 (10.3%) tumors; C-MYC, amplified in 4 of 87 (4.6%) tumors; GATA4, amplified in 9 of 87 (10.3%) tumors; CCND1, amplified in 6 of 87 (6.8%) tumors; DYRK2, amplified in 2 of 87 (2.3%) tumors; HNF3α, amplified in 5 of 87 (5.7%) tumors; and PIK3CA, amplified in 6 of 87 (6.8%) tumors (Fig. 2). Amplification of AIB1, AKT1, and IGF1R was also assessed; however, amplification of these genes was not observed in any adenocarcinoma samples (data not shown). Southern blot analysis verified the results obtained by using Q-PCR (Fig. 3A).

Coamplification of multiple genes was observed in many amplified tumors, with 14 of 50 (28%) tumors containing two amplicons, and 5 of 50 (10%) amplified tumors containing three amplicons. The 8p22 amplicon (GATA4) was frequently coamplified with the ERBB2 gene (5 of 9 tumors; 56%). Analysis of GATA4 and ERBB2 coamplification using Fisher’s exact test indicated a significant association (P = 0.021) between these amplicons. Three cell lines derived from Barrett’s adenocarcinomas in our laboratory were also examined for the presence of gene amplification (Fig. 3B). The immortalized esophageal squamous cell line Het-1A was used as a control. Coamplification of KRAS and C-MYC was detected in the Bic-1 cells. Seg-1 cells demonstrated amplification of C-MYC. Flo-1 cells did not amplify any of the genes examined.

Clinical Characteristics. To determine whether gene amplification was associated with tumor stage and thus associated with events of tumor progression, the presence of one or more gene amplification events and the clinical stage of the tumor were examined (Table 1). Eight of 11 (72%) stage I, 10 of 17 (58%) stage IIA, 4 of 9 (44%) stage IIB, 18 of 37 (48%) stage III, and 10 of 13 (77%) stage IV tumors demonstrated at least one gene amplification event (Table 1). Therefore, gene amplifications were not associated with tumor stage. After ad-
Gene Amplification Frequency in Barrett’s Adenocarcinomas

It remains unclear whether tumors with specific amplicons demonstrate phenotypic characteristics discordant with tumors lacking amplification. PIK3CA gene amplification was the only amplification event that correlated with specific clinical variables in our series of tumors. Amplification of the PIK3CA gene was significantly correlated with early tumor stage (P < 0.0001), small tumor size at resection (P = 0.0163), and the absence of nodal involvement (P = 0.0039), with all amplifications occurring in stage I or IIA tumors. These tumors were also significant for low T status [tumor-node-metastasis (TNM) staging; P = 0.0086], an indication of tumor invasiveness.

Patient records indicated that 62 of 84 (73.8%) patients had a history of smoking, whereas the remaining 22 of 84 (26.2%) patients were nonsmokers, and smoking status was unknown for 3 patients (Table 1). In addition, 34 of 83 (40.9%) patients had a history of alcohol consumption, whereas 49 of 83 (59.1%) patients were nondrinkers, and alcohol status was unknown for 4 patients (Table 1). No association between the occurrence of an amplification event and either smoking or alcohol use was observed.

**Gene Amplification in Barrett’s with HGD.** DNA extracted from 22 Barrett’s with HGD was screened by using Q-PCR for the presence of the 10 amplicons we observed in tumors. Nine of 22 (40.9%) of the HGD specimens had increased gene dosage of one or more genes. Amplification of ERBB2 (1), KRAS (2), C-MYC (1), CCNE1 (2), CCND1 (2), and GATA4 (1) was observed. Fig. 4 demonstrates the observed amplifications for the CCND1, GATA4,

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* Table 1: Association of Barrett’s adenocarcinoma gene amplifications with clinical variables

**Genetic Abnormalities in Barrett’s Adenocarcinoma.** The most commonly amplified genes were ERBB2, CCNE1, and KRAS, with frequencies at or above 21.5%. All other amplicons were nondrivers, and alcohol status was unknown for 4 patients (Table 1). In addition, 34 of 83 (40.9%) patients had a history of alcohol consumption, whereas 49 of 83 (59.1%) patients were nondrinkers, and alcohol status was unknown for 4 patients (Table 1). No association between the occurrence of an amplification event and either smoking or alcohol use was observed.

**Gene Amplification in Barrett’s with HGD.** DNA extracted from 22 Barrett’s with HGD was screened by using Q-PCR for the presence of the 10 amplicons we observed in tumors. Nine of 22 (40.9%) of the HGD specimens had increased gene dosage of one or more genes. Amplification of ERBB2 (1), KRAS (2), C-MYC (1), CCNE1 (2), CCND1 (2), and GATA4 (1) was observed. Fig. 4 demonstrates the observed amplifications for the CCND1, GATA4,

![Gene Amplification Frequency](image)

**Fig. 2** Gene amplification frequencies for Barrett’s adenocarcinoma amplicons. ERBB2 was the most frequently occurring amplicon at 21.5%. Three other amplicons, CCNE1, KRAS, and GATA4, all have frequencies at or above 10%. EGFR, CCND1, and HNF3A were all amplified at frequencies between 5% and 10%. The C-MYC and DYRK2 amplicons were amplified in <5% of tumors.

justing for gender and tumor stage in a proportional hazards model, CCND1 amplification was associated with poorer patient survival (P = 0.044). All other amplicons were unrelated to patient survival (all Ps > 0.09). Kaplan-Meier survival analysis demonstrated that time to death or disease recurrence was strongly dependent on tumor stage (P < 0.01).
and KRAS genes using Q-PCR. Gene dosages for all amplified markers in HGD were between 2- and 4-fold compared with GAPDH, indicating low-level gene amplification or aneuploidy as plausible mechanisms for the observed gene increase. Real-time Q-PCR using GAPDH and GATA4 primers with SYBR Green fluorescent dye confirmed our competitive PCR results in sample G04, indicating an even larger amplification (5.3/H11006 1.8-fold) than detectable by traditional PCR (2.7-fold).

DISCUSSION

Barrett’s adenocarcinomas are often accompanied by a high degree of chromosomal instability. CGH analysis of regional chromosome alterations in Barrett’s adenocarcinomas has revealed DNA copy number changes at many chromosomal locations (32–34). Apart from the current study, however, a thorough analysis of gene amplification has not been performed in a large tumor set. No previous reports have suggested an overall frequency of gene amplification in Barrett’s adenocarcinomas. We identified 57% (50 of 87) of Barrett’s adenocarcinomas as having gene amplification at one or more of the loci tested, indicating a potentially higher frequency if additional amplicons were investigated. Furthermore, 38% of amplified tumors had multiple amplicons, with 10% con-

Fig. 3 A, gene amplification of ERBB2, EGFR, C-MYC, and KRAS assessed by Southern blot analysis of DNA samples from normal esophageal squamous mucosa (N), Barrett’s mucosa (B), normal gastric mucosa (G), and esophageal Barrett’s adenocarcinoma (T). Amplification ratios of ≥2.0 were considered indicative of DNA amplification. Two EcoRI fragments were hybridized with the ERBB2 probe. Tumors from patients S57, H74, and D18 showed high-level ERBB2 gene amplification. Hybridization of EGFR yielded multiple fragments. The tumors from patient R97 exhibited low-level amplification, and those from patient T82 exhibited high-level amplification. KRAS amplification was found in tumors from patients B80, T17, and F74. Hybridization of C-MYC yielded one amplified fragment in tumors from patients D48 and A92. B, gene amplification analysis of ERBB2, EGFR, C-MYC, and KRAS assessed by Southern blot analysis of EcoRI-digested DNA of three Barrett’s adenocarcinoma cell lines (Bic-1, Flo-1, and Seg-1). The cells were derived from primary Barrett’s adenocarcinomas. The nontransformed esophageal squamous cell line Het-1A was used as a control. Flo-1 cells did not demonstrate amplification of these genes. Coamplification of KRAS and C-MYC was detected in the Bic-1 cells. Seg-1 cells demonstrated amplification of C-MYC and a reduced level of the EGFR gene.

Fig. 4 Representative gene amplifications in high-grade dysplastic Barrett’s epithelium assessed by Q-PCR. Two HGD tissues, C07 and S40, demonstrated amplification of KRAS. Tumor G04 amplified the GATA4 gene. Dysplastic Barrett’s specimens from K59 and M00 also had elevated copy number but did not exceed the cutoff value for amplification. CCND1 was amplified in tissues from patients M00 and S65.
taining three or more, indicating that oncogene amplification may play a primary role in effectuating genetic change in Barrett’s adenocarcinomas. Our observation that no significant increase in amplification frequency is seen in advanced tumors compared with stage I tumors further suggests that gene amplification is involved early in the progression to adenocarcinoma rather than representing additional genetic alterations occurring during further tumor development.

The most frequently amplified gene was ERBB2, which was amplified in 19 of 87 (21.8%) adenocarcinomas. ERBB2, a tyrosine receptor kinase located on chromosome band 17q11.2, is also amplified in many other tumor types including breast, brain, and ovarian cancers (35–37). We found that tumors with ERBB2 gene amplification often have high-level ERBB2 mRNA and protein expression (data not shown). Trastuzumab (Herceptin) therapy was shown to significantly prolong survival for patients with ERBB2 overexpression and amplified metastatic breast cancers (38). The ERBB2 gene product may therefore be a useful therapeutic target for a significant fraction of esophageal adenocarcinoma patients.

Amplification and/or overexpression of CCND1, EGFR, and the structurally related ERBB2 gene have been shown to be indicators of poor prognosis in several human cancers (39, 40). The effect of these gene amplifications on patient prognosis with esophageal adenocarcinoma remains equivocal and is complicated by the poor survival of most patients with this disease (1). The best predictor of patient survival was tumor stage ($P < 0.0001$). After adjusting for tumor stage in a proportional hazards model, CCND1 amplification was identified to correlate significantly with poor survival. A similar observation was also reported in Barrett’s metaplasias and Barrett’s adenocarcinomas, where cyclin D1 nuclear staining predicted increased cancer risk and poorer survival among patients with Barrett’s esophagus and adenocarcinoma, respectively (41). Cyclin D1 may therefore provide a target for chemoprevention and/or therapy in a subset of Barrett’s metaplasia and adenocarcinoma patients.

Amplification of PIK3CA (3q26.3) was the only amplicon to correlate strongly ($P < 0.0001$) with stage in our samples because four amplicons occurred in stage I tumors and one amplicon occurred in a stage IIA tumor. Subsequently, amplification of PIK3CA was also associated with absence of nodal involvement ($P < 0.004$), small tumor size ($P < 0.02$), and lower T status ($P < 0.009$). These data may suggest a tendency for tumors with an overly active phosphatidylinositol 3’-kinase pathway to present earlier clinically or demonstrate less invasive characteristics than tumors abrogating alternative pathways.

Esophageal adenocarcinoma is frequently accompanied by tobacco and alcohol abuse, therefore smoking or alcohol consumption may underlie some of the genetic alterations in these tumors. Strong evidence exists for the association of smoking with fragile site expression (42). Induction of fragile sites is proposed to play a significant mechanistic role in the amplification of certain human oncogenes (43). No association was observed, however, between smoking or alcohol consumption and the presence of gene amplification in this study.

The extent to which gene amplification occurs early in esophageal adenocarcinoma progression is currently unclear. In a previous study, Rieger et al. (32) observed infrequent DNA copy number increases at the ERBB2 and EGFR loci in HGD specimens using CGH. Although this approach allows genome-wide analysis of DNA copy number changes, amplicons spanning relatively short distances (200 kb) are often undetectable using CGH. Gene amplification analysis of microdissected regions of Barrett’s mucosa containing HGD identified a relatively high frequency of DNA increases in our Barrett’s samples. In addition to ERBB2, CCNE1, C-MYC, and EGFR, all of which have been reported as amplified in Barrett’s adenocarcinomas with HGD, we identified CCND1, GATA4, and KRAS amplification in HGD lesions (15, 20, 44, 45). Levels of DNA amplification were observed to be much higher in carcinoma tissues (3–22-fold) as compared with HGD samples (2–4-fold). It is currently unclear whether this is a result of enrichment of amplified tissues during tumor growth or a continuation of amplification during tumor development. Combined, these results suggest that gene amplification may initiate during HGD and impel malignant growth. The ability to detect gene amplification in HGD may provide an additional diagnostic (CCND1) tool for identifying Barrett’s patients with high cancer risk. Further understanding of when gene amplification occurs and the mechanisms underlying amplification may therefore prove useful in developing improved treatment regimens for patients with dysplasia and adenocarcinoma.

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REFERENCES


